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Low endogenous NO levels in roots and antioxidant systems are determinants for the resistance of Arabidopsis seedlings grown in Cd

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Running title: NO function under cadmium stress in Arabidopsis seedlings

ABSTRACT

Cadmium (Cd), which is a toxic non-essential heavy metal capable of entering plants and thus the food chain, constitutes a major environmental and health concern worldwide. An understanding of the tools used by plants to overcome Cd stress could lead to the production of food crops with lower Cd uptake capacity and of plants with greater Cd uptake potential for phytoremediation purposes in order to restore soil efficiency in self-sustaining ecosystems. The signalling molecule nitric oxide (NO), whose function remains unclear, has recently been involved in responses to Cd stress. Using different mutants, such as *nia1nia2*, *nox1*, *argh1-1* and *Atnoa1*, which were altered in NO metabolism, we analysed various parameters related to reactive oxygen and nitrogen species (ROS/RNS) metabolism and seedling fitness following germination and growth under Cd treatment conditions for seven days. Seedling roots were the most affected, with an increase in ROS and RNS observed in wild type (WT) seedling roots, leading to increased oxidative damage and fitness loss. Mutants that showed lower NO levels in seedling roots under Cd stress were more resistant than WT seedlings due to the maintenance of antioxidant systems which protect against oxidative damage.

Findings: An excess of endogenous NO produced in plant responses to Cd has a toxic effect, while a finely tuned balance between ROS and NO/RNS is crucial in order to prevent oxidative damage.

INTRODUCTION

The heavy metal Cadmium (Cd) enters the environment essentially due to mining and industrial activity and phosphate fertilizers used in agriculture (Clemens et al., 2013). Cd first enters roots through the cortical tissue and is translocated to above-ground tissues under most environmental conditions. The main problem with regard to its accumulation in plant tissues is its rapid entry into the food chain, which constitutes both an environmental and health hazard worldwide (Clemens et al., 2013). In recent years, much evidence points to an excess of reactive oxygen species (ROS) and oxidative stress as one of the main causes of long-term Cd-related toxicity (Bi et al., 2009; Rodríguez-Serrano et al., 2006; Romero-Puertas et al., 2002). However, ROS, whose toxicity and signalling role are affected by the finely tuned balance between production and detoxification, can act as signalling molecules (Mittler et al., 2011). The free radical nitric oxide (NO), which acts as a ubiquitous inter- and intra-cellular signalling molecule in the regulation of plant development processes, plant defences and responses to stress (León et al., 2016; Sanz et al., 2015; Yu et al., 2014), has recently been found to be involved in plant Cd responses (Besson-Bard et al., 2009; Romero-Puertas et al., 2019). However, contradictory evidence has shown that NO production following exposure to Cd may be time- and dose-dependent (Arasimowicz-Jelonek et al., 2011; Besson-Bard et al., 2009; Romero-Puertas et al., 2012). NO levels appear to peak under short term treatment (Besson-Bard et al., 2008; De Michele et al., 2009; Pérez-Chaca et al., 2014), while NO production was found to decrease under long term treatment in different plant species (Pérez-Chaca et al., 2014; Rodríguez-Serrano et al., 2009; Rodríguez-Serrano et al., 2006; Xiong et al., 2009). On the other hand, it is well established that exogenous pre-treatment with NO protects against Cd toxicity by

64 increasing antioxidant capacity and heavy-metal stress tolerance in plants (Kopyra et al.,
65 2006; Noriega et al., 2007; Terrón-Camero et al., 2019).

66 NO production and the contribution of the different NO sources at any point in
67 the treatment remain unclear and are the subject of much debate in plant biology. A
68 variety of NO production mechanisms in plants, including arginine and hydroxylamine-
69 dependent oxidative pathways, as well as nitrate-dependent reductive pathways, have
70 been described (Mur et al., 2013; Santolini et al., 2017). Thus, apart from non-
71 enzymatic production mechanisms, NO may be generated by a putative nitric oxide
72 synthase-like (NOS-l) protein, which occurs in mammals and generates NO from L-
73 arginine (Astier et al., 2017), and by nitrate reductase (NR) which catalyses the
74 reduction of nitrite to NO (Gupta et al., 2011).

75 Although some NO sources, such as NOS-l and NR, may be involved in NO
76 production in response to Cd (Arasimowicz-Jelonek et al., 2011; Besson-Bard et al.,
77 2008; Pérez-Chaca et al., 2014), their contribution to NO production and their role in
78 plant responses to Cd remain unclear. This paper aims to assess the relationship
79 between antioxidant mechanisms, ROS and RNS production and nitrosative stress in
80 Arabidopsis seedlings, on the one hand, and changes in endogenous NO levels during
81 germination and growth in media caused by Cd on the other. Four pre-characterized
82 mutants were used in this study: two mutants, one impaired in the AtNOA1 (*Atnoa1*)
83 protein and the other in nitrate reductases (NR1/NIA1and NR2/NIA2; *nia1nia2*),
84 previously reported to play a role in NO biosynthesis (Desikan et al., 2002; Modolo et
85 al., 2005; Moreau et al., 2008; Rockel et al., 2002; Yamasaki and Sakihama, 2000); and
86 two NO overproducers, one impaired in the arginase structural gene *ARGH1* (*argh1-1*;
87 Flores et al., 2008) and the other in a chloroplast phosphoenolpyruvate/phosphate

translocator (*nox1/cue1*; He et al., 2004; Streatfield et al., 1999). These four mutants enabled us to characterize endogenous NO-related responses and resistance to Cd stress.

MATERIALS AND METHODS

Plant material, growth conditions and harvesting

Arabidopsis thaliana (Col-0) seeds (WT, *nia1nia2, Atnoa1, nox1* and a*rgh1.1*) were surface-sterilized and stratified for 48 hours at 4ºC. The seedlings were then grown vertically in Hoagland solid medium (0,5x), pH 5.6 (Hoagland and Arnon, 1950) with and without (control) 25 µM cadmium (Cd) in a growth chamber at 22 ºC under 100 µE irradiance, 60-65% relative humidity and 16/8 light/dark conditions for 7 days. When indicated, seedlings were supplemented with NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP, 1 mM; WT, *nia1nia 2*, *Atnoa1*) and NO production inhibitor aminoguanidine (AG, 1mM; WT, *nox1*, *argh1.1*) for 4 days. The plates were scanned to analyse seedling root length. The whole seedlings were then harvested, weighed and processed, and frozen when necessary, for the purposes of enzymatic and non-enzymatic, Western blot and gene expression analysis. Only seedling leaves were used to analyse H_2O_2 and $O_2^{\cdot-}$ with the aid of 3,3′-diaminobenzidine (DAB) and 4-nitro blue tetrazolium chloride (NBT), respectively. The seedling roots were observed under a confocal laser scanning microscope to analyse reactive oxygen and nitrogen species (ROS and RNS) production.

Enzymatic and Western blot analyses

Whole seedlings were homogenized as described elsewhere (Pérez-Chaca et al., 2014). Supernatants were used to measure superoxide dismutase (SOD; EC 1.15.1.1; Beauchamp and Fridovich, 1971), catalase (CAT; EC 1.11.1.6; Aebi, 1984), glycolate oxidase (GOX; EC 1.1.3.1; Kerr and Groves, 1975) and ascorbate peroxidase (APX; EC

1.11.1.11; Jimenez et al., 1997). Ascorbate (1 mM) was added to the extraction buffer to analyse APX activity.

To detect carbonyl groups and nitrated proteins, anti-DNP (1:40000 dilution; Sigma) and anti-nitrotyrosine (N-Tyr; 1:2000 dilution; Sigma) were used as described elsewhere (Romero-Puertas et al., 2002 and Pérez-Chaca et al., 2014, respectively). Protein extracts were incubated with 5 mM sodium dithionite to ensure anti-nitrotyrosine specificity and nitrated BSA was used as positive control (Suppl. Fig. S1). Anti-catalase (1:5000; Agrisera) was used to quantify CAT.

Non-enzymatic assays

A lipid peroxidation assay procedure, as described by Buege and Aust (1978), was used. Cd was localized in plant tissues using diphenylthiocarbazone (650 µM; dithizone; Clabeaux et al., 2011; Seregin and Ivanov, 1997) prepared in acetone (33%) and acetic acid (55%). The samples were stained for 1 hour and then rinsed 3 times with ethanol. Cd-dependent deposits in seedling roots were analysed using a Leica M165FC stereomicroscope.

ROS, NO and peroxynitrite detection

To detect nitric oxide, superoxide radicals, H_2O_2 and peroxynitrite, the seedling roots were incubated with 10 µM 4,5-diaminofluorescein diacetate (DAF-2 DA), 10 µM dihydroethidium (DHE), 25 µM 2'7'-dichlorofluorescein diacetate (DCF-DA) and 25 µM HK2 Green, respectively, as described elsewhere (Rodríguez-Serrano et al., 2006; Sun et al., 2009; Terrón-Camero et al., 2018). Controls were made by pre-incubating samples with 500 µM carboxy-2-phenyl-4,4,5,5-tetramethy-limidaziline-1-oxyl-3-oxide (cPTIO; Suppl. Fig. S2A), a NO scavenger; 1 mM epicatechin (Suppl. Fig. S2B), an $ONOO^-$-specific scavenger (Pannala et al., 1997); 1 mM tetramethyl piperidinooxy (TMP), an $O_2^{\cdot -}$ scavenger (Suppl. Fig. S3B), and 1 mM ascorbate (ASC; Suppl. Fig.

S4B), a H_2O_2 scavenger. Root fluorescence was examined under a confocal laser scanning microscope (Leica TCS). Diphenyliodonium chloride (DPI; 1 mM; Suppl. Fig. S3C) and aminoguanidine (AG; 1 mM; Suppl. Fig. S2C) were used as NADPH oxidase and NOS-like activity inhibitors, respectively. Fluorimetry was used to determine H_2O_2 in whole seedlings as described elsewhere (Romero-Puertas et al., 2004). To detect NO in the whole seedling, extracts were incubated with 20 μM DAF-2 for 2 h at 37 ºC (excitation: 495, emission: 515; Nakatsubo et al., 1998).

Histochemistry and image quantification

A histochemical analysis was carried out to detect $O_2^{\cdot-}$ and H_2O_2 in seedling leaves using NBT (0,1%; w/v) and DAB (1 mg/ml), pH 3,8, respectively, as described elsewhere (Romero-Puertas et al., 2004). Primary seedling root elongation, DAB and NBT staining and fluorescence in seedling root tissue were quantified with the aid of Image J Fiji software (Terrón-Camero et al., 2018). Briefly, average intensity per pixel was analysed in each image with three independent squares per image of the same size.

Reverse Transcription quantitative PCR analysis of gene expression (RT-qPCR)

RNA was isolated from 0,1 g frozen and homogenized seedlings using Trizol reagent (Invitrogen) and DNase according to the manufacturer´s protocol (www.invitrogen.com/content/sfs/manuals/15596026.pdf and Ambion DNA-free kit, respectively). RNA quantification and integrity were verified with the aid of a NanoDrop® ND-1000 spectrophotometer and 1% agarose electrophoresis gel. 1 μg RNA was used as a template for the reverse transcriptase (RT) reaction, and cDNA synthesis was obtained by using a PrimerScript RT reagent Kit (Takara). cDNA was two-fold diluted and stored at -20 ºC for future use. RT-qPCR was performed with the aid of the iCycler iQ Real-Time PCR Detection System (Bio-Rad) using SYBR Premix Ex Taq™ II (https://www.takarabio.com%20Manual/RR820L_e.v1611Da.pdf.). Primer

efficiency was determined by a standard curve using two-fold serial dilution of pooled samples. Primer melting curves with 90-105% efficiency were performed to validate amplification specificity (Suppl. Table S1). Five candidate reference genes, *TUB4* (AT5G44340), *ACT2_1* (AT3G18780_1), *ACT2_2* (AT3G18780_2), *UBI10* (AT4G05320) and *GAPC1* (AT3G04120), were selected from the literature (Calero-Muñoz et al., 2019; Cuypers et al., 2011; Czechowski et al., 2005), and the best combination for normalization was selected using the GrayNorm algorithm (Remans et al., 2014) with expression data from an in-house microarray containing Cd-treated seedlings. Only the *TUB4* gene, whose stability under our conditions in all backgrounds was tested, was selected by the algorithm (Suppl. Fig. S5; Suppl. Table S2). The relative expression of each gene was then normalized using the reference gene selected (*TUB4*), and the results were analysed with the aid of the comparative critical threshold method (Pfaffl, 2001).

Bioinformatic analysis

The dataset containing 26 variables was analysed using hierarchical clustering (Jain et al., 1999; Mitchell, 1997). As a first step, data clusterability was estimated using the Hopkins statistic (Lawson and Jurs, 1990). Hierarchical clustering was performed using Ward distance and the complete linkage method (Jain, 2010; Kaufman et al., 1990; Mitchell, 1997; Taiyun-Wei et al., 2017).

We used the silhouette method (Kaufman et al., 1990) to estimate the number of clusters. The potentially optimal number of clusters was then chosen in order to maximize the average distance between silhouette means (ADSM). We also studied dependencies between the variables as a result of Cd stress in the different mutants. The correlation coefficient between the variables was calculated as Pearson's product-

moment coefficient from previous standard normalization of the variables using the corrplot R package (Taiyun-Wei et al., 2017).

Differences in the quantitative experiments were compared using two-way ANOVA. Mean values for the different treatments were compared using the S-N-K (Student-Newman-Keuls) multiple comparison test ($P<0.05$; Salkind, 2010), by IBM SPPS Statistic 24 and GraphPad Prism 6. Error bars representing standard error median (SEM) are shown in the figures, and the data represented are the mean of at least three independent experiments.

RESULTS AND DISCUSSION

Damage to Cd-treated seedlings

Recent studies of plants, mostly adult when treated, have linked NO to the plant's response to cadmium (Cd), although the mechanisms involved remain unclear. However, the phenotype observed in plants following Cd treatment has common signatures such as growth inhibition (fresh weight and root length) and a redox status imbalance causing oxidative stress (Kolbert et al., 2016; Romero-Puertas et al., 2019; Terrón-Camero et al., 2019). In this study, we analysed how seedlings respond to Cd-related stress during germination and growth, as well as the effect of the different levels of NO present in four pre-characterised mutants and the role played by NO metabolism. Both reductive and oxidative NO-producing pathways in plants are involved in plant responses to Cd stress (Romero-Puertas et al., 2019). Thus, two of the mutants used in this study are directly associated with NO production: *nia1nia2*, which is affected in nitrate reductase activity (reduced to less than 1%), mainly localized in the cytosol (Yamasaki and Sakihama, 2000; Rockel, 2002; Desikan et al., 2002; Guo et al., 2003; Modolo et al., 2005; Moreau et al., 2008) and *argh1-1*, which lacks the arginase gene (mainly localized in the chloroplast and mitochondria), leading to an increase in the

211 NOS-l substrate (L-arginine; Flores et al., 2008). In addition, we used two other mutants
212 in which, though not directly involved in NO production, its levels are modified in the
213 plant: the *Atnoa1* mutant, which lacks the AtNOA1 gene, a cGTPase involved in mRNA
214 translation to proteins in chloroplasts and mitochondria, which shows lower levels of
215 NO under certain conditions such as ABA-dependent stomatal closure and flowering
216 (Moreau et al., 2008); and the *nox1/cue1* mutant, which is altered in a chloroplast
217 phosphoenolpyruvate/phosphate translocator, which shows generally higher levels of
218 NO (Streatfield et al., 1999; He et al., 2004). The contribution of each direct and
219 indirect biosynthetic pathway may fluctuate in different organs in this developmental
220 stage, as the gene expression patterns affected vary according to plant topology as
221 demonstrated by open-access databases (BAR; http://www.bar.utoronto.ca/).
222 Having tested different Cd concentrations (10-50 µM), we selected a Cd
223 concentration of 25 µM. Even though this concentration had visible effects on seedling
224 roots such as decreased root growth, the seedlings were suitable for morphological and
225 biochemical analysis (Suppl. Fig. S6). The effects of lower concentrations of Cd were
226 difficult to quantify, while those caused by higher concentrations were excessively
227 strong in seedlings which barely developed (Suppl. Fig. S6). Three of these mutants:
228 *nia1nia2, Atnoa1* and *nox1*, showed lower fresh weight than that for WT seedlings
229 under control conditions, while *Atnoa1* and *nox1* also recorded lower rates of seedling
230 root growth (Fig. 1). These results are in line with previous characterizations of these
231 mutants. NO-deficient seedlings (*Atnoa1* and *nia1nia2*) showed a decrease in shoot and
232 root growth under normal conditions (Kolbert et al., 2015), and most of which
233 phenotype is reversed when the mutants are supplemented with NO (Lozano-Juste et al.,
234 2010). Similarly, the NO overproducer *nox1/cue1* showed physiological defects
235 including diminished biomass and root growth (He et al., 2004; Frungillo et al., 2014).

This appears to show an absence of correlation between NO levels and root elongation, while the level of NO, which needs to be optimised for normal growth, needs to be strictly regulated (Kolbert et al., 2016). Mutations in the *ARGH1* gene in *argh1-1* mutants, led to an increase in the formation of lateral and adventitious roots whose length appeared to be unaffected (Flores et al., 2008).

Given these results, we compared seedling root length in Cd-treated 7-day-old seedlings with those under control background conditions, as root growth inhibition is one of the earliest marker symptoms of Cd toxicity (Sandalio et al., 2001; Kolbert et al., 2016). A significant decrease in seedling root length was observed in WT plants (49%) and NO-related mutants, with *argh1-1* and *Atnoa1* being more affected (65% and 55%, respectively) and *nia1nia2* and *nox1* less affected (45% and 44%, respectively) than WT plants (Figs. 1A and 1B). Similar decreases in the root length of 7- and 8-day-old Arabidopsis seedlings treated with 30 and 15 μM Cd respect to the non-treated ones (35-55% and of roughly 50%) have been observed in previous studies (Carrió-Seguí et al., 2015; Kim et al., 2006). Fresh weight also decreased under Cd stress conditions in WT plants (46%), being *argh1-1* mutants more affected (57%) and *nia1nia2, Atnoa1* and *nox1* less affected (38%, 39% and 42%, respectively; Figs. 1A and 1C). Although NO levels in mutants and growth inhibition in response to Cd do not appear to correlate, the *nia1nia2* mutant was the least affected while argh*1-1* was the most affected. Under similar conditions, excessive Cu has been shown to inhibit seedling growth in *nox1* mutants to a degree similar to that in WT seedlings, while fresh weight loss was less pronounced in WT seedlings than in nia*1nia2* mutants (Petö et al., 2013). Given the aforementioned complexity of NO metabolism, NO needs to be strictly regulated even under control conditions (Kolbert et al., 2016). In addition, other signalling molecules such as hormones are involved in seedling responses to Cd stress, which might interact

261 with NO. Different levels of NO may also lead to different patterns of NO-dependent
262 post-translational modifications of proteins, such as glyceraldehyde-3-phosphate
263 dehydrogenase, involved in root growth previously (Wang et al., 2017).

264 Oxidative stress is one of the primary effects of Cd treatment in both adult plants
265 and seedlings (Chen et al., 2017; Gupta et al., 2017; Ortega-Galisteo et al., 2012). We
266 have used the whole seedlings to analyse oxidative markers, as roots and leaves at the
267 seedling stage are still too small to be analysed separately. Thus, we observed a Cd-
268 dependent increase in carbonyl group content, a marker of oxidative damage to proteins,
269 in WT seedlings and all the mutants analysed, although *Atnoa1*, which had shown high
270 levels of protein oxidation under control conditions, was unaffected (Fig. 2B). It has
271 been hypothesised that the changes in the levels of NO in *Atnoa1* are caused by
272 defective organelle function. This gives rise to ROS production that may scavenge NO
273 and therefore lower its bioavailability (Moreau et al., 2010; Sudhamsu et al., 2008). An
274 increase in carbonyl content in response to Cd, which depends on its concentration and
275 exposure time, has been observed in Arabidopsis seedlings (Calero-Muñoz et al., 2019)
276 and other species in both root and leaves. In soybean roots, carbonyl content increases at
277 the beginning of treatment with Cd and then declines following longer exposure (Perez-
278 Chaca et al., 2014). Carbonyl content also increases in wheat, tomato, pea and maize
279 leaves (Djebali et al., 2008; Paradiso et al., 2008; Sandalio et al., 2001; Pena et al.,
280 2007) when Cd concentrations are increased. Roots sometimes behave differentially to
281 leaves showing an opposite pattern of oxidation (Djebali et al., 2008) and others, in the
282 same way to leaves (Paradiso et al., 2008). These controversial results probably depend
283 on the developmental stage of the plant, as well as treatment length and dosage
284 (Romero-Puertas et al., 2019).

An increase in lipid damage was also observed in WT seedlings and NO-dependent mutants except for *nia1nia2*, whose lipid integrity appeared to be unaffected in response to Cd (Fig. 2A). *nia1nia2* phenotype is reversed by adding a NO donor (SNAP) to the seedlings, giving rise to a statistically significant increase in NO and lipid peroxidation in this mutant during Cd treatment (Suppl. Figs. S7 and S8). This result suggests that NR-dependent NO is involved, at least in part, in the increase in oxidative damage observed in response to Cd. Actually, lipid peroxidation in *nia1nia2* under control conditions is slightly higher than in WT, probably due to the increased level of NO observed in the seedling roots of the mutant. On the other hand, the increase in lipid peroxidation in response to Cd is four times higher in *argh1-1* than in WT and similar to that in WT in *nox1* seedlings (Fig. 2A). The increase in lipid peroxidation caused by Cd is reduced by 15% in *argh1-1* and by 40% in *nox1* (Suppl. Fig. S8), in line with the decrease observed in NO in the mutants, by adding the NO production inhibitor AG during Cd treatment (Suppl. Fig. S7). These findings further corroborate the involvement of NO in the increase in oxidative damage observed in response to Cd. Most data currently available on lipid peroxidation in response to Cd stress concern adult plants (Romero-Puertas et al., 2019); less is known about seedlings germinated and grown under Cd treatment. Thus, lipid peroxidation has been observed to increase in Cd-treated 6-day-old Arabidopsis seedlings (Chen et al., 2017), which is in line with our findings.

Protein nitration is a major post-translational modification initially associated with nitrosative stress (Radi, 2004), about which little is known in Cd-treated plants. No differences in the total content of nitro-tyrosine (N-Tyr) residues have been found in soybean roots following Cd treatment (Pérez-Chaca et al., 2014), suggesting that Cd induces a finely tuned regulation of protein nitration rather than any major changes. A

later study identified nitrated proteins in soybean under Cd treatment; five of the ten Tyr-nitrated proteins identified were down-regulated during both moderate and intense exposure to Cd, while five were up-regulated only during intense exposure to Cd, with the majority of the identified proteins being associated with proteolysis (Gzyl et al., 2016). While we did not find any significant differences in the pattern of nitrated proteins in seedlings grown under Cd treatment conditions, *Atnoa1* and *nox1* mutants did show a decrease in nitration, although basal levels were higher than those in WT seedlings (Fig. 2C). This result suggests that, as mentioned elsewhere, *Atnoa1* and *nox1* mutants alter RNS-dependent damage even under control conditions (Moreau et al., 2010; Hu et al., 2014).

NO mutants showed altered ROS and RNS metabolism under Cd stress

When analysing the whole seedling, no changes in total H_2O_2 (Fig. 3D) and a decrease in total NO levels were observed in WT seedlings (Fig. 5C). We then analysed the different parts of the seedling and observed a decrease in ROS (H_2O_2 and $O_2^{\cdot-}$) production with the aid of DAB and NBT histochemistry in WT seedling leaves (Fig. 3A, 4A and Suppl. Figs. S3A and S4A). Using CLSM, we observed an increase in ROS/RNS (H_2O_2, $O_2^{\cdot-}$ and $ONOO^-$) in WT seedling roots (Figs. 3B and 3C; Figs. 4B and 4C; Figs. 5D and 5E). Thus, under the conditions studied, oxidative stress and fitness loss in the seedling appears to have been caused by an increase in ROS/RNS production in seedling roots and, to a lesser extent, in seedling leaves. At this stage, the small size of seedling leaves may affect seedling responses. Consequently, the damage observed in WT seedlings grown under Cd treatment occurred concurrently with the increase in H_2O_2, $O_2^{\cdot-}$ and $ONOO^-$ in 7-d-old WT seedling roots (Figs. 3B and 3C; Figs. 4B and 4C; Figs. 5D and 5E). ROS production has previously been shown to increase in different species including Arabidopsis in response to Cd, especially under long-term

335 treatment conditions, both in adult plants (Gupta et al., 2017; Ortega-Villasante et al.,
336 2007; Pérez-Chaca et al., 2014; Rodríguez-Serrano et al., 2006) and in seedlings (Kulik
337 et al., 2012). H_2O_2 production has been shown to increase in the roots of 3-week-old
338 Arabidopsis plants treated with 50 µM Cd (Bahmani et al., 2019), while H_2S prevented
339 this increase in 2-week-old seedling roots (Jia et al., 2016). However, in adult plants
340 growing for three weeks under normal conditions and then treated with low
341 concentrations of Cd, an increase in H_2O_2 was observed in both leaves and roots
342 (Cuypers et al., 2011).

343 We observed a decrease in total NO in WT seedlings in response to Cd (Fig.
344 5C), with no changes being observed in seedling roots (Figs. 5A and B). This suggests
345 that, although an initial increase in NO is usually observed in plant responses to Cd
346 stress (Balestrazzi et al., 2009; Bartha et al., 2005; Groppa et al., 2008; Mahmood et al.,
347 2009; Pérez-Chaca et al., 2014; Valentovičová et al., 2010), probably associated with
348 signalling, NO levels are brought under control at a later stage (Terrón-Camero et al.,
349 2019). Thus, NO has been shown to be involved in reducing root growth in plant
350 responses to Cd in different species (Groppa *et al.*, 2008; Besson-Bard *et al.*, 2009;
351 Valentovičová *et al.*, 2010) by repressing auxin accumulation and signalling in
352 *Arabidopsis* plants (Yuan and Huang, 2016). Longer treatments might also affect NOS-l
353 activity due to a Ca deficiency and decreasing NO levels (Rodriguez-Serrano et al.,
354 2009), and to an induced early senescence (McCarthy et al., 2001; Rodriguez-Serrano et
355 al., 2009).

356 The increase in ROS and RNS production observed in WT seedling roots was
357 concomitant with a decrease in antioxidant enzymes, such as ascorbate peroxidase
358 (APX) and catalase (CAT), which maintain ROS homeostasis (Fig. 6). CAT activity,
359 which may be responsible for the bulk removal of excess H_2O_2 produced under stress

conditions (Mittler, 2002), is affected in Cd-treated seedlings grown, decreasing its activity by more than 50% (Fig. 6B). *CAT2* gene expression also fell by over 50% and the protein almost disappeared (Figs. 6C and D). Several studies have analysed APX activity under Cd stress, mainly in adult plants, which generally respond via increased APX activity in roots and leaves, especially under short-term treatment with Cd (Cuypers et al., 2016, 2011; Pérez-Chaca et al., 2014; Terrón-Camero et al., 2019). As with APX, a number of studies have shown that CAT activity and/or gene expression under Cd stress conditions are dependent on tissue, as well as species and length of the treatment, without, at times, any direct relationship between CAT activity and gene expression (Cuypers et al., 2016; Romero-Puertas et al., 2019; Terrón-Camero et al., 2019). For example, in three-week-old Arabidopsis plants, changes in CAT activity under Cd stress conditions depend on the plant tissue and concentrations of the metal (Cuypers et al., 2011), while, in five-week-old plants, CAT activity increases in leaves after 1- and 5-d treatments (Gupta et al., 2017). However, in pea leaves, as in our study, following a long high-dose treatment, this activity was found to decrease (Sandalio et al., 2001).

In our study, ROS and NO production in NO mutants differed with respect to responses to Cd and, at times, under control conditions, in relation to one another and with respect to WT plants, suggesting, as described elsewhere, that there is intense crosstalk between NO and ROS (Lindermayr and Durner, 2015; Romero-Puertas and Sandalio, 2016). Thus, overproduction of NO in seedling roots (Fig. 5A and 5B) in response to Cd, a decrease in H_2O_2 (Figs. 3B and C) and no changes in $O_2^{\cdot -}$ and $ONOO^-$ (Figs. 4B and 4C and Figs. 5D and 5E, respectively) were observed in *argh1-1* and *nox1* mutants. Both these mutants have been found to increase the arginine substrate for NOS-l activity (Flores et al., 2008; He et al., 2004), suggesting that, as described

385 elsewhere, the arginine-dependent pathway is involved in increasing seedling root NO
386 production under Cd stress (Rodríguez-Serrano et al., 2009; Besson-Bard et al., 2009).
387 Increased NO content in seedling roots may also directly or indirectly affect other
388 antioxidant systems lowering H_2O_2 levels in these mutants. However, the mutants
389 *argh1-1* and *nox1* behave in very similar way to WT plants in relation to oxidative
390 damage and down-regulation of antioxidant activity. These results suggest that the
391 excess of NO observed in *argh1-1* and *nox1* mutants and disturbances in ROS
392 homeostasis caused by the down-regulation of antioxidant enzymes induce oxidative
393 damage. The two antioxidants analysed in our study are regulated by NO-dependent
394 post-transcriptional modifications (PTMs) in different species (Begara-Morales et al.,
395 2014; Correa-Aragunde et al., 2013; de Pinto et al., 2013), while CAT is specifically
396 regulated by oxidation and *S*-nitrosylation under Cd stress conditions in pea leaves
397 (Ortega-Galisteo et al., 2012; Romero-Puertas et al., 2002). Although low levels of NO
398 may boost the antioxidant system, especially under Cd stress conditions, excessive NO
399 has the potential to become toxic to plants (Romero-Puertas et al., 2019).

400 Surprisingly, *nia1nia2* mutants showed higher levels of NO in seedling roots
401 under control conditions (Fig. 5A and 5B), which decreased after treatment with the
402 NOS inhibitor AG (Suppl. Fig. S2C). This suggests that NOS-l activity may be involved
403 in increasing the level of NO in *nia1nia2* mutants, indicating that there is crosstalk
404 between the different sources of NO in plants. Other studies have suggested that the
405 different NO production pathways interact with one another and that nitrate reductase
406 (NR) regulation in response to high levels of CO_2 is NOS-l-dependent (Du et al., 2016;
407 Romero-Puertas and Sandalio, 2016). We found that Cd treatment reduced the level of
408 NO in *nia1nia2* seedling roots to that observed in WT seedling roots under control
409 conditions, while an opposite pattern was observed in the NOS-l related mutants *argh1-*

410 *1* and *nox1* (Figs. 5A and 5B). Although the level of $O_2^{\cdot -}$ was found to decrease in
411 *nia1nia2* mutants under control conditions and to increase following Cd treatment, it
412 was similar to that recorded in WT seedlings under control conditions (Figs. 4B and
413 4C). The decrease in NO production following Cd treatment in *nia1nia2* mutants was
414 found to coincide with a decrease in $ONOO^-$, caused by the reaction of $O_2^{\cdot -}$ and NO
415 (Fig. 5D-E; Koppenol et al., 1992). In addition, H_2O_2 was observed to decrease in
416 *nia1nia2* after treatment with Cd (Figs. 3B and 3C), probably due to the maintenance
417 and apparent protection of CAT activity (Figs. 6B and 6C). This result suggests that NR
418 plays a role in preventing the down-regulation of CAT activity under Cd stress
419 conditions, although the mechanisms involved, apart from the above-mentioned NO-
420 dependent PTMs, need further investigation.

421 Despite the apparent effect of stress on *Atnoa1* mutants in our study and its
422 impact on seedling development described elsewhere (Moreau et al., 2008; Sudhamsu et
423 al., 2008), we did not find any differences in ROS/RNS production in WT seedlings
424 which was not found to differ under Cd stress as compared to control conditions (Figs. 3
425 and 5). As mentioned previously, an imbalance in ROS metabolism has been described
426 in these mutants, and, while the NOA1 protein may play a role in ROS/RNS production
427 under Cd stress conditions, the mechanism involved needs to be studied in greater
428 depth. Interestingly, APX activity in the *Atnoa1* mutant was unaffected by Cd treatment
429 (Fig. 6A), suggesting that NOA1 is involved in decreasing this activity.

430 As previously shown in different species, incubation with diphenyliodonium
431 (DPI) suggests that NADPH oxidases and possibly peroxidases (Rodríguez-Serrano et
432 al., 2006) were involved in $O_2^{\cdot -}$ production (Suppl. Fig. S3C) under Cd stress conditions
433 (Gupta et al., 2017; Pérez-Chaca et al., 2014; Rodríguez-Serrano et al., 2006; Ros-
434 Barcel, 1999). It is interesting to note that treatment with DPI also decreased H_2O_2

production after Cd treatment (Suppl. Fig. S3C), suggesting that this was mostly due to the spontaneous dismutation of the $O_2^{\cdot -}$ radical, as SOD activity and gene expression were found to decrease with Cd treatment in our experiments (Fig. 7). The Cu,Zn-SOD isoforms showed the highest levels of activity, accounting for 85 to 93% of the total activity depending on background and conditions (Suppl. Fig. S9B). We observed a decrease in Cu,Zn-SOD activity in response to Cd in all genotypes except for *argh1-1* and *Atnoa1* mutants (Fig. 7A and Suppl. Fig. S9B). On the other hand, the plastidic gene *Cu,Zn-SOD2* was down-regulated in all genotypes (Fig. 7) under Cd stress conditions, suggesting that NO was not involved in regulating this gene. Similarly, the *Cu,Zn-SOD2* gene was down-regulated in three-week-old Arabidopsis leaves under Cd stress conditions for 24h (Smeets et al., 2009). Interestingly, a down-regulation of the peroxisomal *Cu,Zn-SOD3* gene was also observed in *nia1nia2* mutants (Fig. 7D), suggesting that NR may regulate this isoenzyme under Cd toxicity conditions. However, to the best of our knowledge, the molecular mechanism involved in this process has not been elucidated and requires further analysis. Mn- and Fe-SOD activities account for 7 to 15% of the total activity in our conditions suggesting that these activities may have played a less important role than Cu,Zn-SOD. Though difficult to detect in gel, we quantified a decrease in the percentage of Mn- and Fe-SOD activities under Cd treatment conditions (Suppl. Fig. S9C). To our knowledge, no data exist on SOD activity in seedlings germinated and grown under Cd treatment conditions, while the various outcomes of plants and seedlings grown under normal conditions, which were then treated with Cd, have been the subject of considerable study. Total SOD activity has been observed to increase in wheat and sunflower leaves, as well as in Arabidopsis seedlings (DalCorso et al. 2008; Laspina et al., 2005; Song et al., 2012) and to decrease in wheat and bean (Milone et al., 2003; Cardinaels et al., 1984), with no significant

effect found in Arabidopsis seedlings (Smeets et al. 2009; Cuypers et al. 2011). Different SOD isoforms have also been observed to behave differentially, with Cu,Zn-SOD activity decreasing under Cd stress conditions in pea leaves and roots and Arabidopsis leaves (Sandalio et al., 2001; Rodríguez-Serrano et al., 2006; Gupta et al., 2017), and increasing in Arabidopsis seedlings and soybean roots (Abozeld et al., 2017; Pérez-Chaca et al., 2014). Mn-SOD activity appears to be less affected by Cd in pea leaves (Sandalio et al., 2001), with no changes observed in pea roots or Arabidopsis seedlings (Rodríguez-Serrano et al., 2006; Abozeld et al., 2017). Fe-SOD activity, which also behaves differentially depending on the age, species and strength of the treatment, increases in Cd-treated Arabidopsis seedlings (Abozeld et al., 2017) while it decreases and remains unaffected in pea leaves and roots, respectively (Sandalio et al., 2001; Rodríguez-Serrano et al., 2006).

Glycolate oxidase (GOX), a key photorespiration enzyme associated with peroxisomes, has been reported to contribute to Cd-dependent ROS production in Arabidopsis and pea plants after long-term treatment (Gupta et al., 2017; Romero-Puertas et al., 1999) and also in soybean (Pérez-Chaca et al., 2014). We therefore analysed GOX activity, which was found to decrease in WT seedlings following Cd treatment. No significant changes were observed in NO-related mutants, except for *argh1-1*, which recorded an increase in GOX activity, suggesting that GOX is regulated differently in this mutant (Suppl. Fig. S9).

Cd uptake in NO mutants

Cd is known to be able to enter the food chain through plant roots and then to translocate to above-ground tissues (Shahid et al., 2016). After using dithizone to precipitate Cd, similar levels of the metal were accumulated in seedling leaves for all genotypes, except for *nia1nia2*, which accumulated two-fold more than WT seedlings

(Figs. 8A and 8B). Few Cd precipitates were observed in *nia1nia2* seedling roots as compared to WT seedlings (Figs. 8A and 8C), suggesting that translocation of Cd is induced in this mutant, a process that requires further investigation. Half of the Cd-dependent precipitates in the WT seedling roots were found in *nox1* mutant seedling roots, while in argh*1-1* accumulated more Cd (Figs. 8A and 8B). The entry of Cd into the root is well known to be partly due to the IRT1 iron transporter (Clemens et al., 2013) whose gene expression is considerably inhibited by Cd treatment, probably in order to prevent metal accumulation (Connolly, 2002; Connolly et al., 2003). Though down-regulated in Cd-treated WT seedlings, the *IRT1* iron transporter was up-regulated in *nox1* mutants which had higher levels of NO (Fig. 8D). Although *IRT1* was not upregulated in *argh1-1*, *IRT1* gene expression was significantly higher in this mutant than in WT seedlings under control conditions, which is in line with the higher level of Cd accumulation observed in roots (Fig. 8C and 8D). Lower expression of *IRT1* in *nox1* mutant is in accordance with the lower Cd accumulation found in *nox1* seedling roots. This result pointed to the possibility that other mechanism, beyond NO, are involved in the regulation of *IRT1* in *nox1* mutants under control conditions. On the other hand, *IRT1* was not repressed in *nia1nia2* or *Atnoa1*, suggesting that NR and NOA1 play a role in its regulation, a mechanism which requires further investigation (Fig. 8D). It has been shown that down-regulation of *IRT1* intensified in plants co-treated with Cd and a NOS inhibitor (Nω-Nitro-L-arginine methyl ester hydrochloride; L-NAME), suggesting that NO is involved in the up-regulation of *IRT1* under Cd stress (Besson-Bard et al., 2009). Therefore, NO needs to be tightly controlled in plant responses to Cd, as endogenous NO may increase metal uptake. Thus, overexpression of *Nicotiana tabacum* haemoglobin (*NtHb1*) in both tobacco and *Arabidopsis* plants, which down-regulate NO in response to Cd, was recently found to increase Cd tolerance, partly due to lower

metal accumulation (Lee and Hwang, 2015; Bahmani *et al*., 2019). Furthermore, HY1 has been shown to increase Cd tolerance in Arabidopsis plants by reducing NO production and enhancing Fe homeostasis (Han et al., 2014).

Differential responses of NO-related mutants and WT seedlings to Cd stress

To gain more insight into WT seedling and NO-related mutant responses to Cd, we performed a clustering analysis of all the parameters analysed under control and Cd treatment conditions. We obtained two clusters under normal conditions: one cluster contained WT seedlings and NO-related mutants, except for *nia1nia2,* which was in the other cluster (Suppl. Figs. S10A and S10B). In the first cluster obtained under control conditions, the *Atnoa1* mutant exhibited the most differential behaviour. However, under Cd treatment conditions, we obtained five clusters being each background in its own cluster. Comparing the clusters obtained under Cd conditions, *nia1nia2* again exhibit the most differential behaviour and *nox1* behave more like WT seedlings (Suppl. Figs. S10C and S10D). We also performed a correlation analysis of all the parameters studied. We found that Cd treatment of WT seedlings closely correlated with oxidative damage to proteins and lipids, ROS and peroxynitrite production in seedling roots and an expected accumulation of Cd in seedling roots and leaves (Fig. 9A). By contrast, we observed a negative correlation of Cd with seedling biomass, ROS production in seedling leaves and in the whole seedlings. GOX and SOD activity, as well as CAT and APX, also correlated negatively with Cd. Finally, Cd exhibited a negative correlation with *IRT1* gene expression (Fig. 9A). The main difference between the *nia1nia2* mutant and WT seedlings was the oxidative damage to lipids which were unaffected in the mutant and being this behaviour reversed by NO donors. GOX activity was positively correlated with Cd in *nia1nia2* mutant. However, H_2O_2 and RNS production in seedling roots in *nia1nia2* were characterised by a trend opposite to that in WT seedlings, in

535 which the regulation of CAT activity in response to Cd was also altered (Fig. 9B).
536 Superoxide and RNS remained unchanged in *Atnoa1* mutants in response to Cd stress,
537 resulting in a negative correlation between Cd and protein damage (Fig. 9C). ROS and
538 RNS production was altered in *nox1* mutants in response to Cd as compared to WT
539 seedlings, while nitration increased significantly. Unlike in WT seedlings, *IRT1*
540 expression correlated positively with Cd in *nox1* mutants (Fig. 9D). The *argh1-1*
541 seedlings behaved differently from WT seedling roots with respect to ROS and RNS
542 production, while SOD activity was not so affected (Fig. 9E).

543 **Concluding remarks**

544 We observed an increase in ROS (H_2O_2 and $O_2^{\cdot-}$) and RNS ($ONOO^-$) in Cd-
545 treated WT seedling roots as compared to control conditions, which, in addition to
546 inhibiting the antioxidant system, induced oxidative stress as demonstrated by an
547 increase in carbonyl groups and lipid peroxidation (Fig. 10); both these markers
548 correlated positively with Cd (Fig. 9A). This oxidative stress probably led to a reduction
549 in the root length and fresh weight of Cd-treated seedlings, parameters that negatively
550 correlated with the metal (Fig. 9A). Each NO-related mutant and possibly the source of
551 NO affected respond differently to Cd, not only with respect to NO production but also
552 in relation to ROS metabolism. The increase observed in ROS production in WT
553 seedling roots grown under Cd conditions did not occur in *nox1* and *argh1-1* mutants.
554 Nevertheless, the increased levels of NO in *argh1-1* and *nox1* mutants and the decline in
555 the antioxidant system appear sufficient to induce oxidative damage, leading to a
556 decrease in seedling growth. The decrease in the level of NO in response to Cd in
557 *argh1-1* and *nox1* mutants, caused by the incubation with the NO production inhibitor
558 AG, mitigates the increase in lipid peroxidation in these mutants, correlating NO with
559 oxidative damage (Fig. 10). The *Atnoa1* mutants did not vary ROS and RNS production

in seedling roots following Cd treatment. However, these smaller seedlings appeared to be subject to oxidative stress under control conditions, as total H_2O_2 content, lipid peroxidation, carbonyl group and nitration levels were higher than in WT seedlings, suggesting that *Atnoa1* mutants may not be appropriate option for future research in this field. Nevertheless, given that ascorbate peroxidase (APX) in *Atnoa1* mutant grown under Cd conditions was unaffected, NOA1 could provide APX protection leading to lower protein nitro-oxidative damage. Therefore, under Cd conditions, nitration declines and carbonyl groups remain at levels similar to those under control conditions in *Atnoa1* mutants (Figs. 9 and 10). The mutant least affected by Cd was *nia1nia2*. In this mutant, NO and $ONOO^-$ production fell in seedling roots with the treatment, while total H_2O_2 content and lipid peroxidation remained at levels similar to those under control conditions. Therefore, in *nia1nia2* mutant a lower decrease in seedling fresh weight was observed. Catalase (CAT) in the *nia1nia2* mutant was not down-regulated by Cd treatment. The protection of CAT together with the reduction in Cd accumulation in seedling roots may be responsible for the phenotype observed in *nia1nia2*. An excess of endogenous NO appears to be detrimental to *nox1* and *argh1-1* mutants under Cd stress conditions, while the maintenance of the antioxidant system is crucial in order to prevent oxidative damage. A finely tuned balance between ROS and NO/RNS therefore appears to be necessary to regulate responses to Cd-treated seedlings.

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586 **Figure Legends**

587 **Fig. 1: Effect of Cd on Arabidopsis seedling phenotype and fitness.** WT seedlings, as
588 well as *nia1nia2*, *Atnoa1*, *nox1* and *argh1-1* mutants, were germinated and grown in
589 Hoagland medium, with and without (control) 25 µM Cd. Pictures of seedlings (A),
590 seedling root length (B) and fresh weight (C) after 7 days are shown. Different letters
591 denote significant differences as determined by the Student-Newman-Keuls test
592 ($P<0.05$).

593 **Fig. 2: Effect of Cd on lipids and proteins of Arabidopsis seedlings.** (A) Lipid
594 peroxidation measured as malondyaldehyde (MDA) content; (B) protein oxidation
595 measured as carbonyl group content and (C) nitration level determined by Western blot.
596 Different letters denote significant differences as determined by the Student-Newman-
597 Keuls test ($P<0.05$).

598 **Fig. 3: Effect of Cd on H_2O_2 accumulation in Arabidopsis seedlings.** Quantification
599 of images (Suppl. Fig. S4A) with H_2O_2-dependent precipitates in seedling leaves by
600 DAB staining (A); (B) representative images of H_2O_2-dependent fluorescence in
601 seedling roots and quantification (C); total H_2O_2 content (D) in the whole seedlings
602 grown or not under Cd conditions for 7 days. Different letters denote significant
603 differences as determined by the Student-Newman-Keuls test ($P<0.05$).

604 **Fig. 4: Effect of Cd on $O_2^{\cdot-}$ accumulation in Arabidopsis seedlings.** Quantification of
605 images (Suppl. Fig. S3A) with $O_2^{\cdot-}$-dependent precipitates in seedling leaves by NBT
606 staining (A); (B) representative images of $O_2^{\cdot-}$-dependent fluorescence in seedling roots
607 grown or not (control) under Cd conditions for 7 days and quantification of the images
608 (C). Different letters denote significant differences as determined by the Student-
609 Newman-Keuls test ($P<0.05$).

610 **Fig. 5: Effect of Cd on NO and $ONOO^-$ accumulation in Arabidopsis seedlings.** (A)
611 Representative images of NO-dependent fluorescence in seedling roots and image
612 quantification (B); total NO content determined by fluorimetry using DAF2-DA (C) in
613 seedlings grown or not (control) under Cd conditions for 7 days. (D) Representative
614 images of $ONOO^-$-dependent HKGreen-2 fluorescence in seedling roots and image

quantification (E). Different letters denote significant differences as determined by the Student-Newman-Keuls test ($P<0.05$).

Fig. 6: Effect of Cd on antioxidant enzymes in Arabidopsis seedlings. APX (A) and CAT (B) activities; *CAT2* gene expression (C) and catalase content (D) by Western blot in seedlings grown or not (control) under Cd conditions. Different letters denote significant differences as determined by the Student-Newman-Keuls test ($P<0.05$).

Fig. 7: Effect of Cd on superoxide dismutase in Arabidopsis seedlings. (A) Quantification by Image J software, of Cu,Zn-SOD activity. Total SOD activity was carried out by native gels with seedlings grown or not (control) under Cd conditions (shown in Suppl. Fig. S9B). (B, C, D) Analysis of *Cu,Zn-SOD1, Cu,Zn-SOD2* and *Cu,Zn-SOD3* gene expression in seedlings under control and Cd treatment. Different letters denote significant differences as determined by the Student-Newman-Keuls test ($P<0.05$).

Fig. 8: Cd accumulation in Arabidopsis seedlings. Representative image of Cd-dependent precipitates in seedling leaves and roots (A) and quantification of the images by the Image J software (B, C). *IRT1* gene expression (D) in seedlings grown or not (control) under Cd conditions. Different letters denote significant differences as determined by the Student-Newman-Keuls test ($P<0.05$).

Fig. 9: Analysis of data by bioinformatics. Coefficient of correlation between variables in WT (A), *nia1nia2* (B), *Atnoa1* (C), *nox*1 (D) and *argh1-1* (E) was calculated as the Pearson's Product Moment Coefficient previous standard normalization of the variables using the corrplot R package.

Fig. 10: Scheme for NO functions in Arabidopsis seedlings expose to Cd. Arabidopsis seedlings grown in Cd showed an increase in ROS (H_2O_2 and $O_2^{\cdot -}$) and RNS production in WT seedling-roots while a decrease in ROS is observed in seedling-leaves. The increase in ROS seedling-roots is accompanied by an inhibition of the antioxidant system, such as catalase (CAT) and ascorbate peroxidase (APX). Nitrate reductase (NR) appears to be involved in CAT inhibition while NOA1 in the inhibition of APX. The excess of ROS in roots and the imbalance in the antioxidant system induces an oxidative damage in both, proteins and lipids being this one due in part to NR-NO dependent. On the other hand, an increase of L-arg-dependent endogenous NO also induces an increase in lipid peroxidation. Oxidative damage observed lead to a decrease in seedling fitness.

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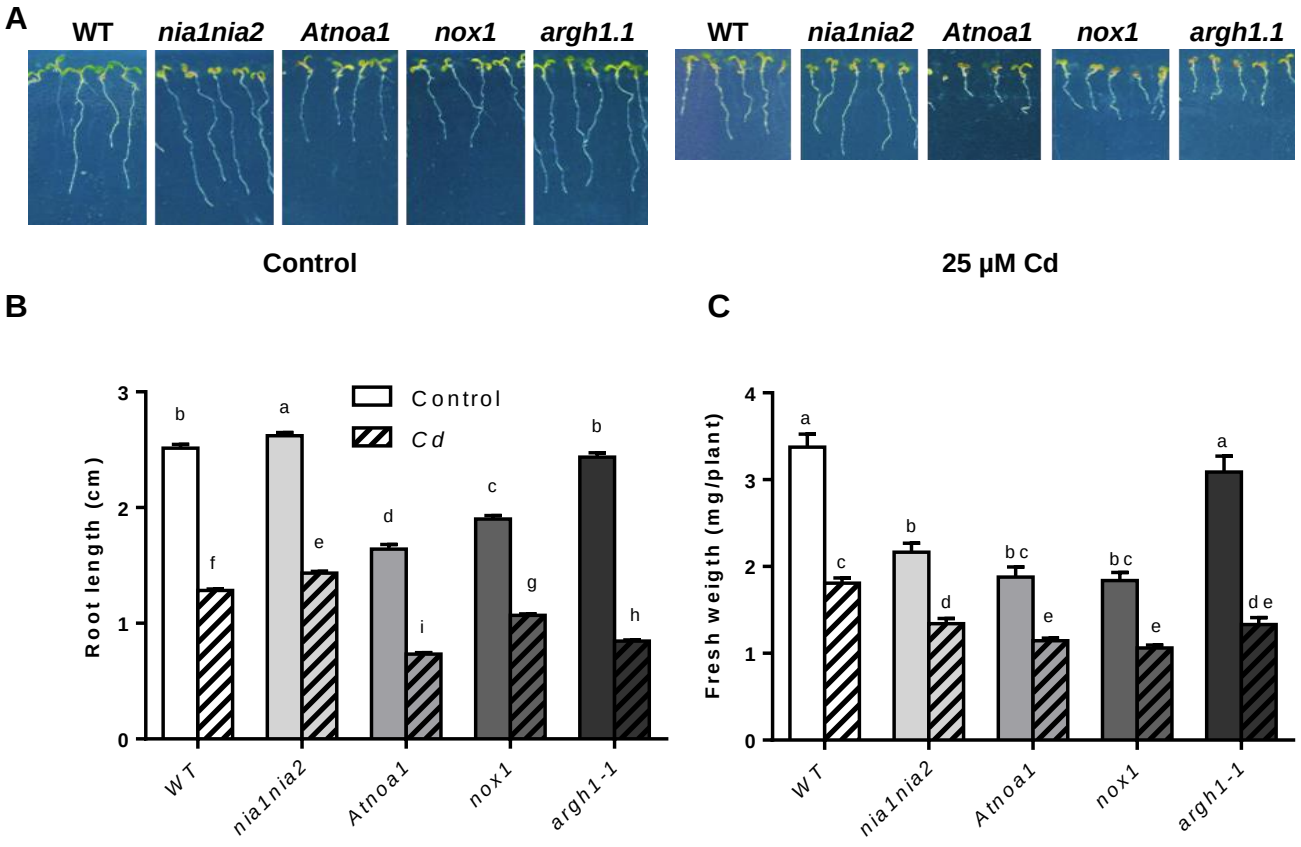
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WT
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argh1.1
Control
25 µM Cd
B
Root length (cm)
Control
Cd
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Fresh weigth (mg/plant)
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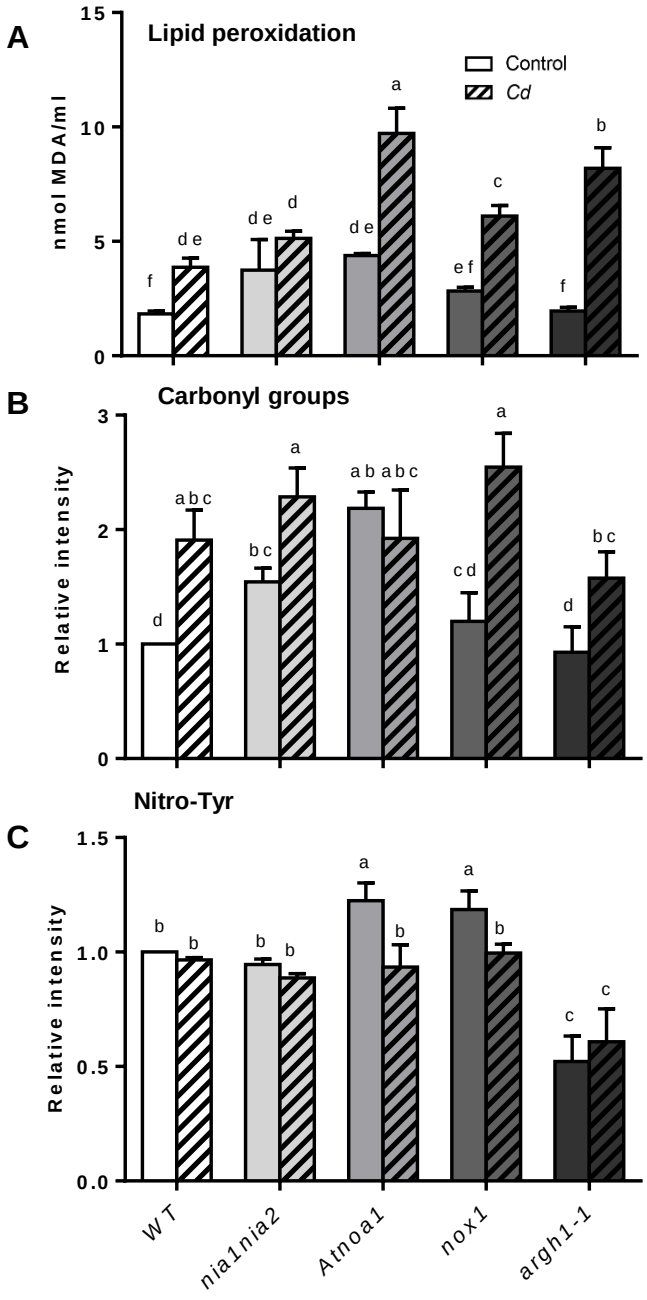
Fig.1


A
Lipid peroxidation
Control
Cd
nmol MDA/ml
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5
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d e
d e
d
d e
a
e f
c
f
b
B
Carbonyl groups
Relative intensity
3
2
1
0
d
a b c
b c
a
a b a b c
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d
b c
C
Nitro-Tyr
Relative intensity
1.5
1.0
0.5
0.0
b
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b
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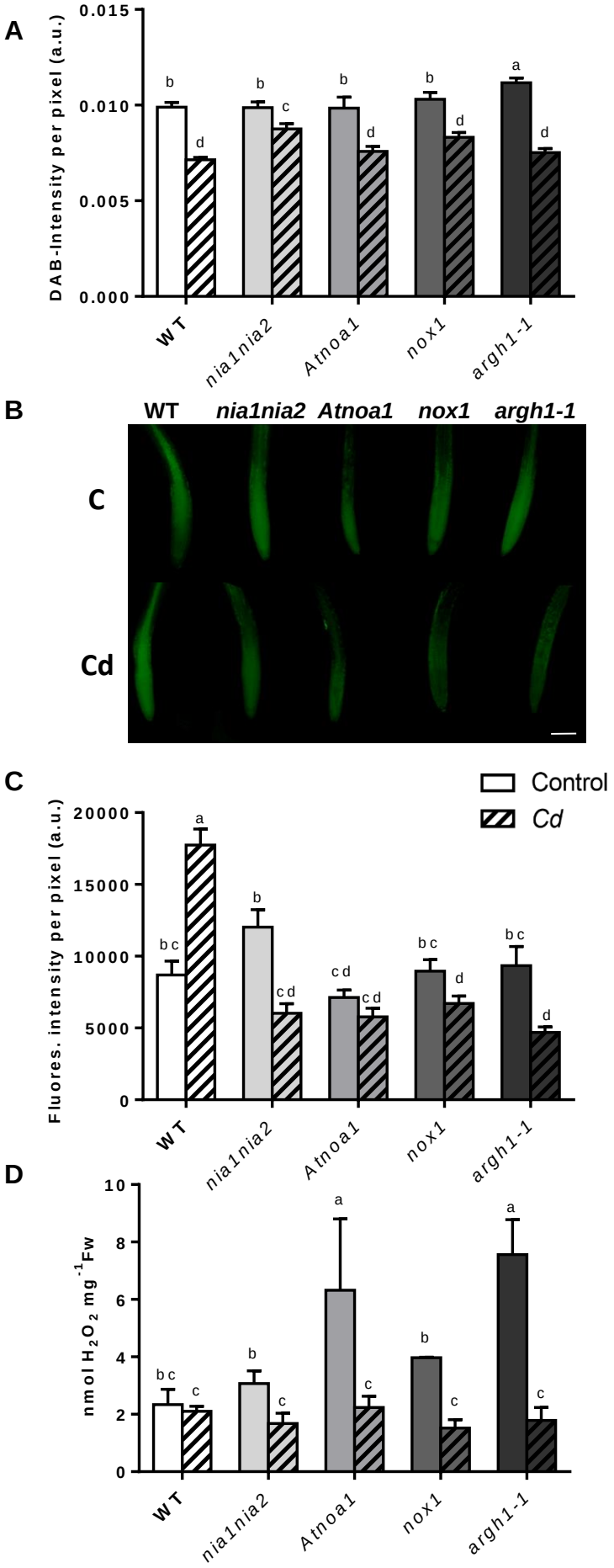
Fig. 2

Fig. 3


A
DAB-Intensity per pixel (a.u.)
0.015
0.010
0.005
0.000
b
d
b
c
b
d
b
d
a
d
WT
nia1nia2
Atnoa1
nox1
argh1-1
B
WT
nia1nia2
Atnoa1
nox1
argh1-1
C
Cd
C
Control
Cd
Fluores. intensity per pixel (a.u.)
20000
15000
10000
5000
0
b c
a
b
c d
c d
c d
b c
d
b c
d
WT
nia1nia2
Atnoa1
nox1
argh1-1
D
nmol H2O2 mg-1Fw
10
8
6
4
2
0
b c
c
b
c
a
c
b
c
a
c
WT
nia1nia2
Atnoa1
nox1
argh1-1

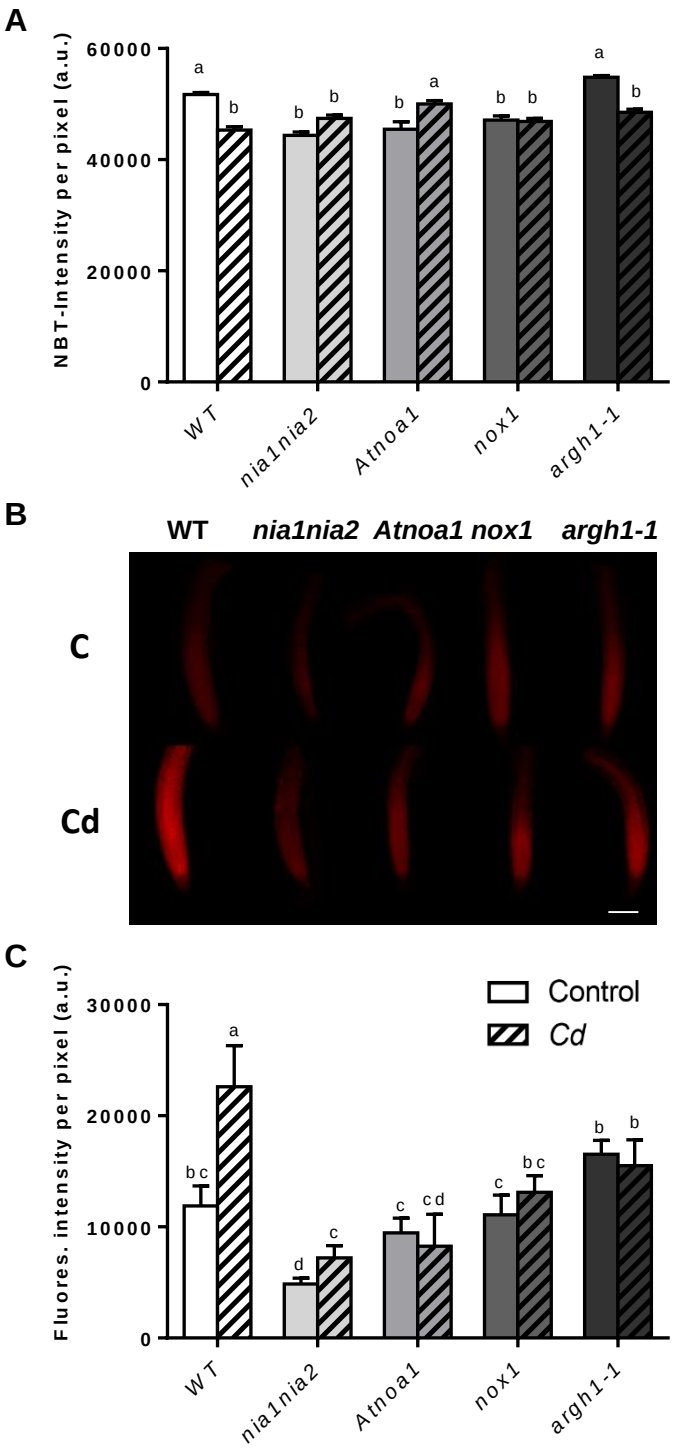
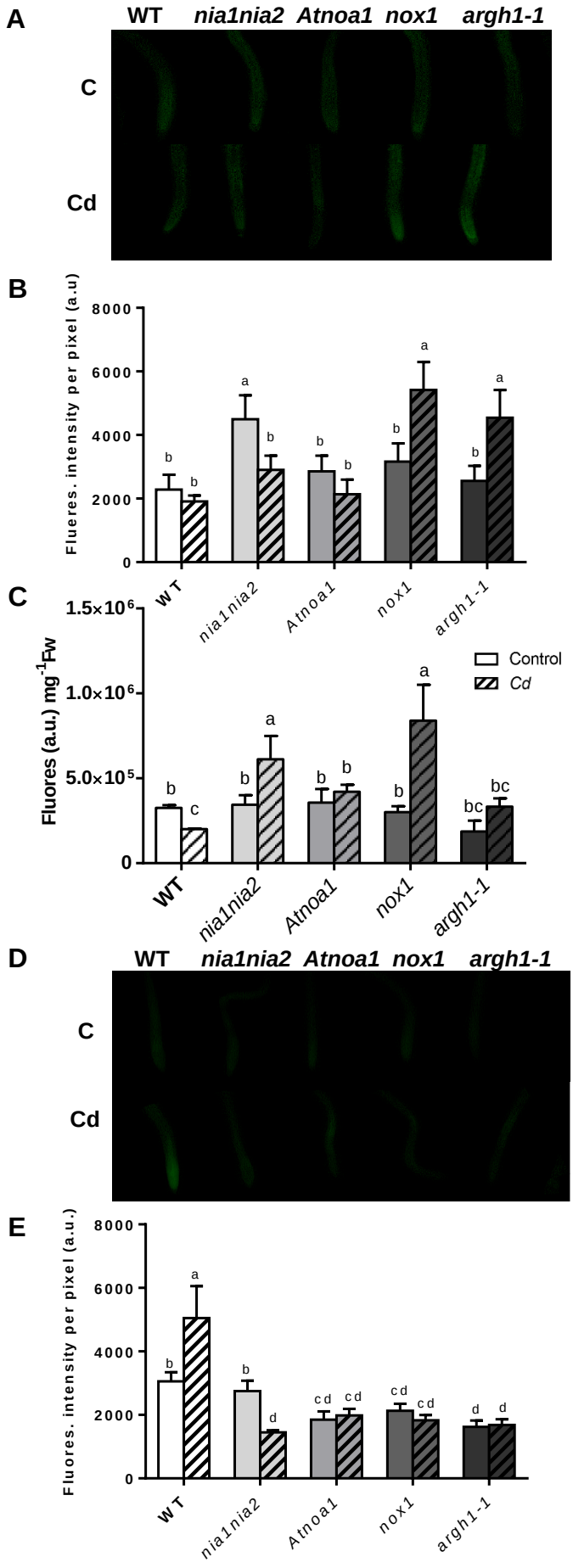

A
NBT-Intensity per pixel (a.u.)
60000
40000
20000
0
a
b
b
b
b
a
b
b
a
b
WT
nia1nia2
Atnoa1
nox1
argh1-1
B
WT
nia1nia2
Atnoa1
nox1
argh1-1
C
Cd
C
Fluores. intensity per pixel (a.u.)
30000
20000
10000
0
Control
Cd
b c
a
d
c
c
c d
c
b c
b
b
WT
nia1nia2
Atnoa1
nox1
argh1-1

Fig. 4

Fig. 5


A
WT
nia1nia2
Atnoa1
nox1
argh1-1
C
Cd
B
Fluores. intensity per pixel (a.u)
8000
6000
4000
2000
0
WT
nia1nia2
Atnoa1
nox1
argh1-1
C
Fluores (a.u.) mg-1Fw
1.5×10⁶
1.0×10⁶
5.0×10⁵
0
Control
Cd
D
E
Fluores. intensity per pixel (a.u.)

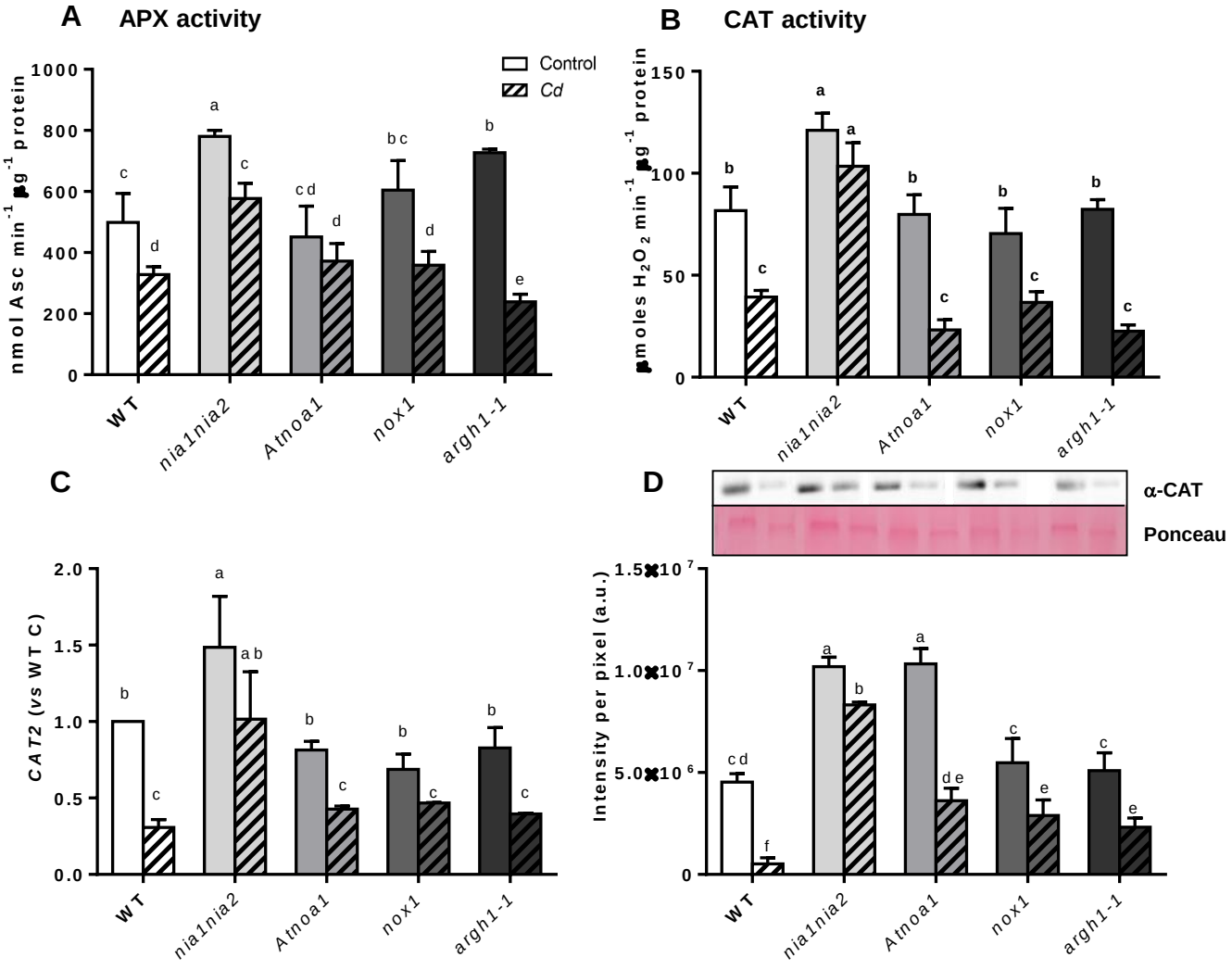

A
APX activity
Control
Cd
nmol Asc min-1 µg-1 protein
1000
800
600
400
200
0
WT
nia1nia2
Atnoa1
nox1
argh1-1
B
CAT activity
µmoles H2O2 min-1 µg-1 protein
150
100
50
0
C
CAT2 (vs WT C)
2.0
1.5
1.0
0.5
0.0
D
α-CAT
Ponceau
Intensity per pixel (a.u.)
1.5✖10 7
1.0✖10 7
5.0✖10 6
0

Fig. 6

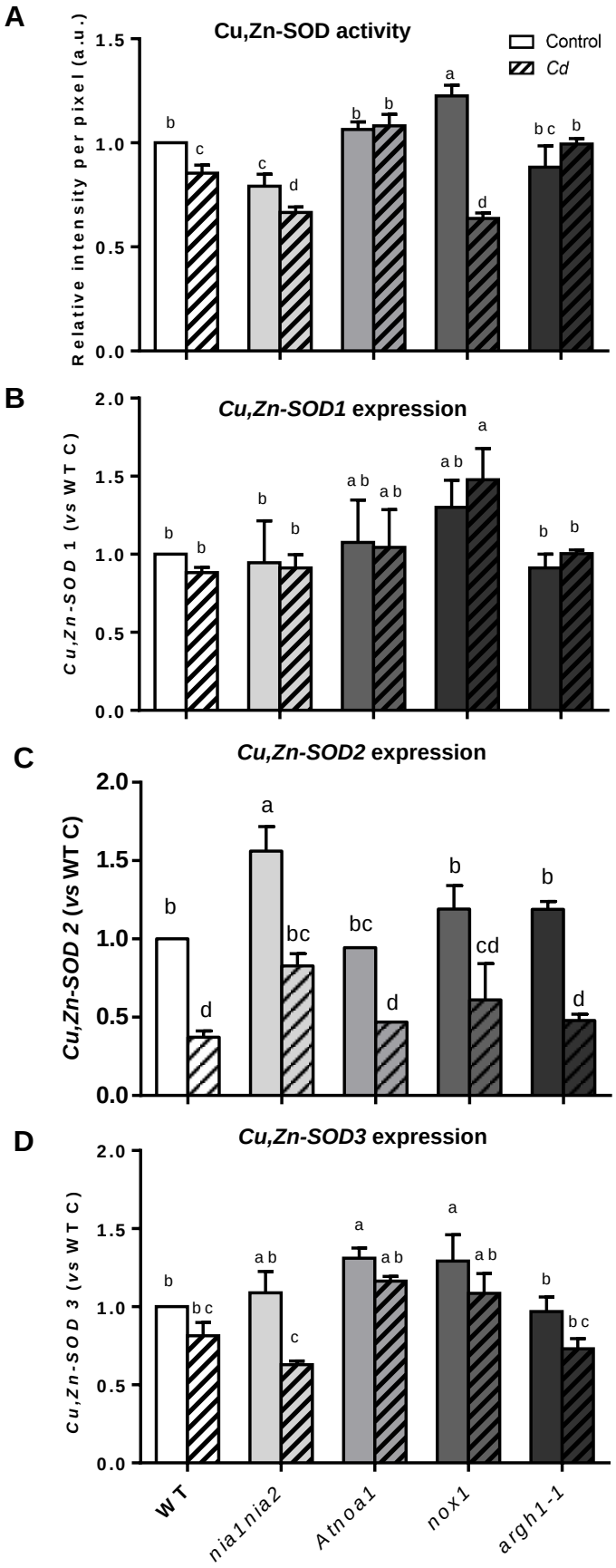

A
Cu,Zn-SOD activity
Control
Cd
Relative intensity per pixel (a.u.)
1.5
1.0
0.5
0.0
b
c
c
d
b
b
a
d
b c
b
B
Cu,Zn-SOD1 expression
Cu,Zn-SOD 1 (vs WT C)
2.0
1.5
1.0
0.5
0.0
b
b
b
b
a b
a b
a b
a
b
b
C
Cu,Zn-SOD2 expression
Cu,Zn-SOD 2 (vs WT C)
2.0
1.5
1.0
0.5
0.0
b
d
a
bc
bc
d
b
cd
b
d
D
Cu,Zn-SOD3 expression
Cu,Zn-SOD 3 (vs WT C)
2.0
1.5
1.0
0.5
0.0
b
b c
a b
c
a
a b
a
a b
b
b c
WT
nia1nia2
Atnoa1
nox1
argh1-1

Fig. 7

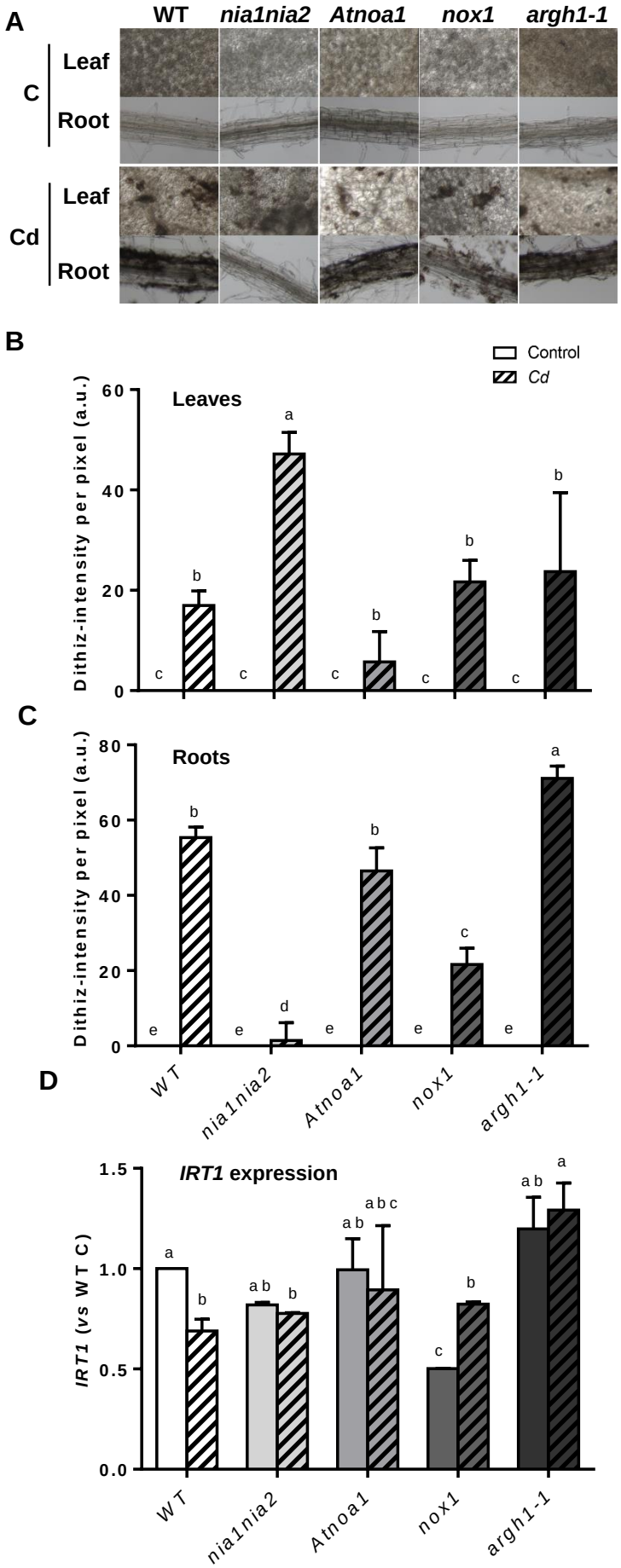

A
WT
nia1nia2
Atnoa1
nox1
argh1-1
C
Leaf
Root
Cd
Leaf
Root
B
Control
Cd
Leaves
Dithiz-intensity per pixel (a.u.)
C
Roots
Dithiz-intensity per pixel (a.u.)
WT
nia1nia2
Atnoa1
nox1
argh1-1
D
IRT1 expression
IRT1 (vs WT C)
WT
nia1nia2
Atnoa1
nox1
argh1-1

Fig. 8

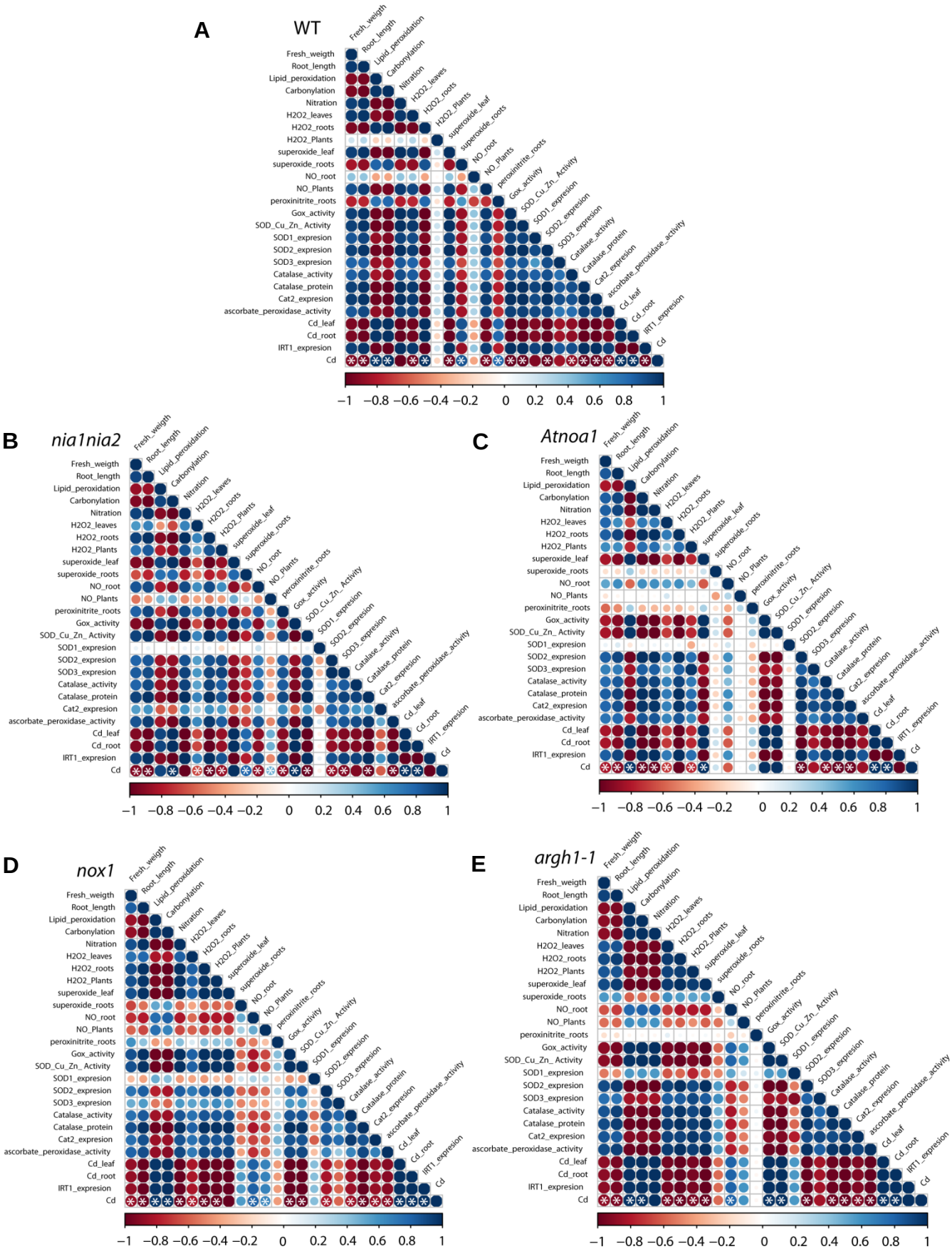

A
WT
B
nia1nia2
C
Atnoa1
D
nox1
E
argh1-1
Fresh_weigth
Root_length
Lipid_peroxidation
Carbonylation
Nitration
H2O2_leaves
H2O2_roots
H2O2_Plants
superoxide_leaf
superoxide_roots
NO_root
NO_Plants
peroxinitrite_roots
Gox_activity
SOD_Cu_Zn_ Activity
SOD1_expresion
SOD2_expresion
SOD3_expresion
Catalase_activity
Catalase_protein
Cat2_expresion
ascorbate_peroxidase_activity
Cd_leaf
Cd_root
IRT1_expresion
Cd
−1 −0.8 −0.6 −0.4 −0.2 0 0.2 0.4 0.6 0.8 1

Fig. 9

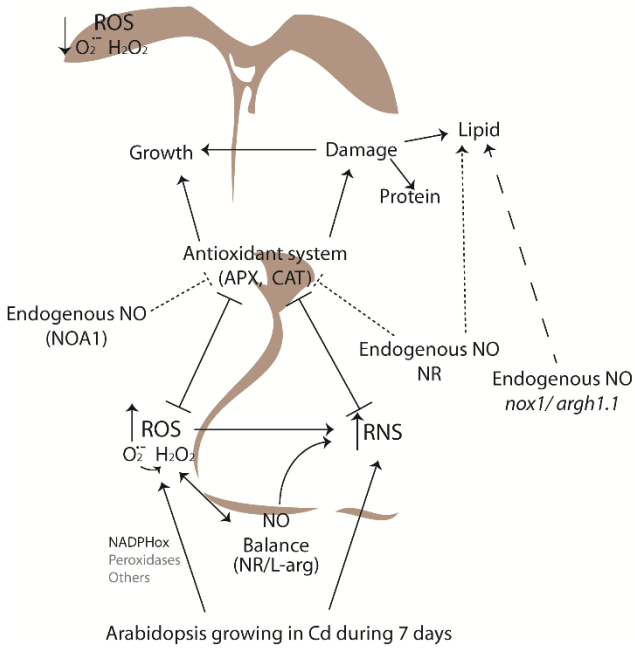
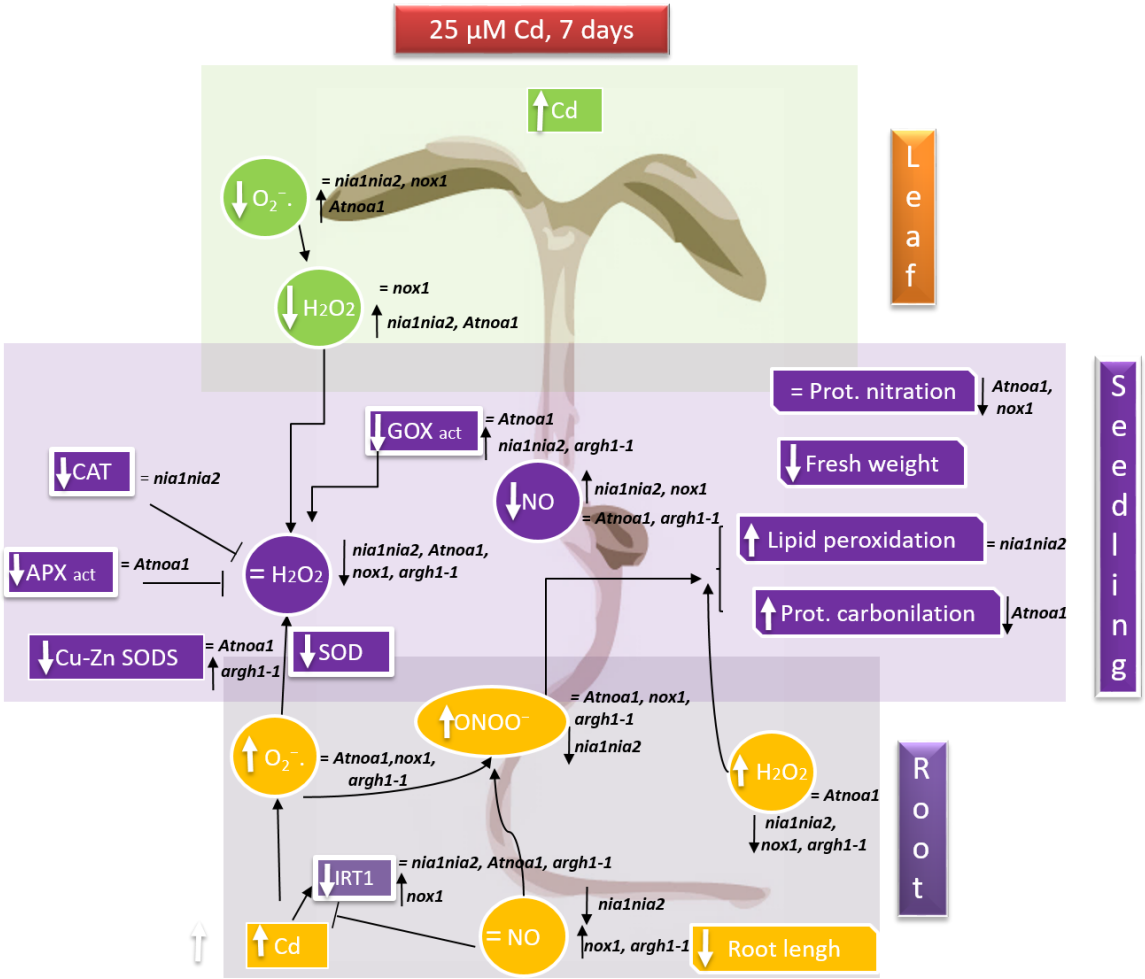

ROS
$O_2^{\cdot-}$ H_2O_2
Growth
Damage
Lipid
Protein
Antioxidant system
(APX, CAT)
Endogenous NO
(NOA1)
Endogenous NO
NR
Endogenous NO
nox1/ argh1.1
ROS
$O_2^{\cdot-}$ H_2O_2
RNS
NO
Balance
(NR/L-arg)
NADPHox
Peroxidases
Others
Arabidopsis growing in Cd during 7 days

Fig. 10



Graphical Abstract

Scheme showing reactive oxygen and nitrogen species production, oxidative damages and seedling fitness of WT, *nia1nia2*, *Atnoa1*, *nox1* and *argh1-1*, germinated and grown in cadmium.